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TITLE	(54): ANTI-GM-CSF AUTOANTIBODY AND MEASURING REAGENT THEREFOR
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/2*

aim 1] An anti-granulocyte macrophage colony-stimulating
utoantibody having the following characteristics: (1) the
nulocyte macrophage colony-stimulating factor autoantibody is
from the bronchoalveolar lavage fluid or blood serum of
with idiopathic pulmonary alveolar proteinosis; and (2) the
nulocyte macrophage colony-stimulating factor is able to
proliferation of human peripheral blood monocytes and the
ll strain TF-1, but the interleukin-3 is unable to inhibit
iferation of human peripheral blood monocytes and the tumor
ain TF-1.

aim 2] A reagent for measuring the autoantibody described in
in a human specimen, wherein the reagent contains the anti-
yte macrophage colony-stimulating factor.

aim 3] The measuring reagent described in Claim 2, wherein the
is an idiopathic alveolar proteinosis diagnostic agent.

aim 4] A method for measuring the autoantibody described in
in a human specimen, wherein the human specimen is reacted
anti-granulocyte macrophage colony-stimulating factor or
r thereof, and the reaction products are measured.

d Description of the Invention]

Industrial Field of Application]

present invention relates to a specific autoantibody for
nulocyte macrophage colony-stimulating factor (GM-CSF) and a

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[0005] In other words, the present invention is an anti-granulocyte macrophage colony-stimulating factor (GM-CSF) autoantibody having the following characteristics: (1) the anti-granulocyte macrophage colony-stimulating factor autoantibody is obtained from the bronchoalveolar lavage fluid (BALF) or blood serum of patients with idiopathic pulmonary alveolar proteinosis (IPAP); and (2) the GM-CSF is able to inhibit proliferation of human peripheral blood monocytes and the tumor cell strain TF-1, but the interleukin-3 (IL-3) is unable to inhibit the proliferation of human peripheral blood monocytes and the tumor cell strain TF-1. The present invention is also a reagent for measuring the GM-CSF autoantibody in a human specimen, wherein the reagent contains the GM-CSF. In addition, the present invention is a method for measuring the GM-CSF autoantibody in a human specimen, wherein the human specimen is reacted with the GM-CSF or indicator thereof, and the reaction products are measured.

[0006] [Embodiment of the Invention]

The anti-GM-CSF autoantibody of the present invention is obtained from the bronchoalveolar lavage fluid (BALF) or blood serum of patients with idiopathic pulmonary alveolar proteinosis (IPAP). In extracting the anti-GM-CSF autoantibody from BALF, the protein fraction is obtained from the BALF in the usually manner, the protein fraction is bonded with GM-CSF, and the bonded fraction is extracted. In detecting the fraction bonded to GM-CSF, ELISA should be utilized with GM-CSF and labeled mouse GM-CSF antibodies. The refinement of the anti-GM-CSF autoantibodies can be performed by combining ordinary

protein refining means with ELISA. For example, butanol processing, a cationic ion-exchange resin column (MonoS column), an anionic ion-exchange resin column (MonoQ column), a gel filtration column (Superrosel12 column), an anionic ion-exchange resin column (ResourseQ column) and a cationic ion-exchange resin column (ResourseS column) can be combined with ELISA to extract the proteins bonded to the GM-CSF.

[0007] The anti-GM-CSF autoantibody of the present invention is obtainable from the BALF of IPAP patients who account for more than 90% of adult-onset pulmonary aveolar proteinosis cases. It is not obtainable from the BALF of secondary PAP patients and healthy persons.

[0008] The anti-GM-CSF autoantibody of the present invention inhibits both the propagation of human peripheral blood monocytes and tumor cell strain TF-1 in the presence of GM-CSF. In other words, human peripheral blood monocytes and tumor cell strain TF-1 are known to propagate when cultivated in the presence of GM-CSF, but the propagation of both is inhibited when the anti-GM-CSF autoantibody of the present invention is also present in the cultivation system.

[0009] IL-3 is functionally equivalent to GM-CSF, and both human peripheral blood monocytes and tumor cell strain TF-1 propagate in the presence of IL-3. However, the propagation of neither is inhibited when the anti-GM-CSF autoantibody of the present invention is also present in the cultivation system. Therefore, the autoantibody of the present invention clearly bonds specifically to GM-CSF.

[0010] The autoantibody of the present invention prevents the boding of GM-CSF to TF-1 only at certain concentrations. In other /3
words, the bonding of GM-CSF to TF-1 was not prevented during a test in which rinsed TF-1 was used after premixing the autoantibody of the present invention with the TF-1. Clearly, the autoantibody of the present invention acts not on the TF-1 or monocytes but on the GM-CSF itself to prevent propagation of both cells.

[0011] The autoantibody of the present invention also has the following characteristics.

(3) The autoantibody of the present invention inhibits bonding of GM-CSF to anti-GM-CSF monoclonal antibodies. In the bonding inhibitory testing, for example, GM-CSF ELISA such as rat anti-GM-CSF monoclonal antibodies were rendered in solid phase, the GM-CSF was reacted with the autoantibody of the present invention and then with tagged mouse anti-GM-CSF antibodies, and the bonded tagged amount was measured.

[0012] (4) The autoantibody of the present invention is not broken down by tripsin, lysozyme or protease K. In other words, the autoantibody of the present invention does not lose its ability to bond with GM-CSF even after interacting with tripsin, protease K or lysozyme.

[0013] (5) The autoantibody of the present invention is inactive after heat processing for 10 minutes at 100°C, chloroform processing or methanol processing, but stable after heat processing for 30 minutes at 55°C, n-butanol processing, EDTA processing or 2-

mercaptoethanol processing. In other words, the autoantibody of the present invention remains stable even after processing for 30 minutes at a pH between 4 and 11.

[0014] (6) The molecular weight of the autoantibody of the present invention according to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is 180 kD under non-reduction conditions and 57 kD under reduction conditions.

[0015] (7) The autoantibody of the present invention is in the immunoglobulin fraction. The isotype is also IgG.

[0016] The autoantibody of the present invention bonds specifically with GM-CSF to control, it is believed, the various actions of GM-CSF. Therefore, the autoantibody of the present invention can be used in the treatment of inflammatory diseases in which excessive production of GM-CSF is observed, such as cutaneous allergies, asthma, chronic rheumatoid arthritis, posttransplant immunoreactions, idiopathic pneumonitis and idiopathic pulmonary alveolar proteinosis. These diseases, particularly idiopathic pulmonary alveolar proteinosis, can be diagnosed by measuring the autoantibody of the present invention in the human specimen.

[0017] In measuring the autoantibody of the present invention, GM-CSF should be used. In other words, the autoantibody is reacted with GM-CSF or an indicator in the human specimen, and the reaction product is measured. The measuring method can be any immunological measuring method common in the art. For example, a turbidimetric immunoassay or labeled immunoassay can be used. For the labeled

immunoassay, a radioimmunoassay, enzyme immunoassay or phosphorescent immunoassay can be used. The human specimen can be BALF, blood plasma or blood serum. The GM-CSF label can be a radioisotope such as 125I, 3H or 14C, an enzyme such as peroxidase or alkaliphosphatase, or a compound such as biotin or digoxigenin.

[0018] The reaction of the human specimen with the GM-CSF or label should be started by bringing the human specimen into direct contact with the GM-CSF or label, bringing the human specimen into contact with the GM-CSF or label in solution, or bringing the human specimen into contact with solid-phase GM-CSF or a label.

[0019] Examples of reaction product measuring means include measuring the reaction product directly, aggregating the autoantibody and GM-CSF complex in the reaction product (latex bead aggregation method, Ouchterlony method, or reacting a secondary antibody with the human specimen (ELISA method). This measurement can also be performed by measuring the GM-CSF activity decrease due to the GM-CSF reaction with the autoantibody. The GM-CSF activity decrease can be measured by reacting the GM-CSF with the autoantibody in the presence of TF-1 cells and then measuring the rate of propagation for the TF-1 cells due to the GM-CSF.

[0020] The autoantibody of the present invention can be measured in any human specimen, but the presence of nearly all of the autoantibody of the present invention in the blood serum of patients with idiopathic pulmonary alveolar proteinosis when measured provides

a particularly sensitive and specific blood serum diagnoses of idiopathic pulmonary alveolar proteinosis.

[0021] [Working Examples]

The following is a detailed explanation of the present invention with reference to working examples. The present invention is by no means restricted to these working examples.

[0022] Working Example 1 (Anti-GM-CSF Autoantibody Preparation)

(1) Reagents

The recombinant GM-CSF (specific activity: 2.25 x 10^8 U/mg) was provided by Kirin Beer Co., Ltd. The recombinant IL-3 was purchased from R&D Systems Co., Ltd. The rat anti-GM-CSF monoclonal antibodies were provided by Associate Professor Kitamura at the Institute of Medical Science [University of Tokyo] (Kitamura, T. et al, J. Cell. Physiol. 140:323-334). The peroxidase-labeled anti-GM-CSF monoclonal antibodies were purchased from R&D Systems Co., Ltd. The radioactive iodide-treated recombinant human GM-CSF was purchased from NEN Life Science Products Co., Ltd. The RPMI 1640 cultures were purchased from Nissui Co., Ltd. The HiTrapS, HiTrapQ, Sephacryl 2000, ResourceQ and ResourceS columns were purchased from Pharmacia Biotech Co., Ltd.

[0023] (2) Subjects

Bronchoalveolar lavage fluid was taken from 11 IPAP patients, /4
2 secondary PAP patients and from healthy non-smokers. The pulmonary proteinosis diagnosis was performed using a biological analysis of the BALF and a histopathological diagnosis of lung specimens. The medical histories and current symptoms of IPAP patients were examined in

detail to make sure no other diseases were present. A chronic myelocytic leukemia examination was performed on both secondary PAP patients. Informed consent was obtained from all of the subjects in writing.

[0024] (3) Bronchoalveolar Lavage Fluid Supernatant (BALF)

The bronchoalveolar lavage fluid was centrifuged for 15 minutes at 1000 x g. The resulting supernatant was centrifuged for another 60 minutes at 40,000 x g, and the supernatant was extracted (hereinafter referred to as BALF). The protein concentration was measured using the Bardford method.

[0025] (4) Segregation of Human Peripheral Blood Monocytes

The human peripheral blood monocytes were obtained from the blood serum of healthy subjects using the Ficoll-Paque specific gravity centrifugation method. This was re-suspended in an RPMI 1640 culture, added to a plastic flask coated beforehand with 10% AB inactive blood serum, cultivated for 15 minutes at 37°C in a CO_2 environment, and the unattached cells were rinsed five times in PBS and removed. After treating the attached cells for 10 minutes in tripsin/PBS, they were collected using a cell scraper (Sumitomo Bakelite). The cells obtained in this manner were determined to be more than 98% monocyte from the form they took and from non-specific esterase dying.

[0026] (5) Cultivation of TF-1 Cells

GM-CSF, IL-3 and erythropoietin-dependent TF-1 tumor cells were cultivated in RPMI 1640 with 10% FCS and 10 ng/ml GM-CSF present.

Before use, the cells were rinsed four times in PBS and the GM-CSF was removed.

[0027] Measuring Cultivation of the TF-1 Cells and Monocytes

The propagation of TF-1 cells and monocytes was measured using an MTT assay. The monocytes (1 x 10^4/well) and TF-1 cells (2 x 10^4/well) were placed in a 96-hole plate and cultivated for three days in the presence of various concentrations of GM-CSF, IL-3 (0, 12.5, 25, 50, 100 ng/ml) and BALF (0, 12.5, 25, 50, 100ng/ml) from IPAP patients or healthy subjects. After an examination under a phase-contrast microscope, 5 μg/ml MTT (3-[4,5-dimethylazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Co., Ltd.) was added. After two hours of cultivation, the formazan formations due to the live cells were dissolved in 100 μl of isopropanol/HCl and the absorptivity at OD 550 nm was measured.

[0028] (7) Measuring the Receptor Bonding of Radioactive Iodide-Labeled GM-CSF

The bonding to the GM-CSF receptors was performed in accordance with the method of S. Chiba et al. (Leukemia 4:29-36). First TF-1 cells (5 x 10^5) were suspended in a 1 ml RPMI 1640 culture and reacted for 90 minutes at 15°C with 150 pM radioactive iodide-labeled GM-CSF and BALF from IPAP patients and healthy subjects at various concentrations. The background reaction was prepared by adding an excess amount of non-radioactive GM-CSF to the same reaction solution. The cell suspension was centrifuged for five minutes at 250 x g, rinsed three times, and measured for radioactivity using a γ counter.

[0029] (8) Measuring Bonding Inhibition of GM-CSF and Antibodies Using the Enzymatic Antibody Method (ELISA)

The bonding inhibition between GM-CSF and the antibody were measured using GM-CSF ELISA. A micro ELISA plate was coated with 100 µl of 0.5 µg/ml anti-GM-CSF monoclonal antibodies (23B6) and allowed to stand overnight at 4°C. After rinsing five times with PBS, a blocking reagent (Stabilicoat, BSI Co., Ltd.) was added and treatment was performed for one hour at room temperature. After removing the blocking reagent, a reaction was performed for two hours at room temperature in the various wells using a mixture of 50 µl 25 ng/ml GM-CSF and 50 µl of BALF at various concentrations (0-500 g/mg). After rinsing in PBS, peroxidase-labeled anti-human GM-CSF polyclonal antibodies were added to each well, and a reaction was performed for one hour at 37°C. Pigmentation was performed using a TMB solution (Color Reagents, R&D Co., Ltd.). The absorptivity at 450 nm was measured using microplate spectrophotometer Model 3550 (Biorat Co., Ltd.). TNF, IL6 and MCP-1 were performed using commercially available assay kits. The percent inhibited (%) was determined using the following formula.

Percent Inhibited (%) = [1 - Detected GM-CSF (ng/ml)/25 (ng/ml)] x 100

[0030] (9) Detection of Bonded Proteins Using the Cross-Linking Method

IPAP and healthy BALF (500 µg/ml) were reacted at room temperature for 90 minutes in a bonding buffer for radioactive iodide-labeled GM-CSF (25 mM HEPES, pH7.4, 150mM NaCl, 10mM KCl, 10mM $CaCl_2$).

In the control, the reaction was performed the same way except that 100 times the mount of non-radioactive GM-CSF was added. The cross-linking reaction was performed for 15 minutes at 4°C by adding 300 μM disuccinimidylsuberate (DSS). The reaction was stopped by adding 1 ml of quenching buffer (50mM Tris-HCl pH8.0, 15mM NaCl, 2mMEDTA). SDS-PAGE was performed on the reaction solution, the gel was dried, and autoradiography was performed.

[0031] (10) Refining GM-CSF Bonded Proteins From IPAP Patient BALF

Next, 400 ml of distilled water-saturated n-butanol was added to the same amount of IPAP BALF, and the mixture was shaken vigorously for five minutes. After being allowed to stand for 10 minutes, centrifugation was performed 3000 times in 10 minutes and the aqueous layer was removed. After performing this operation three times, dialysis was performed on the collected aqueous layer using ultra pure water and then the layer was freeze-dried. After dissolving the freeze-dried proteins in 20 mM ammonium acetate pH 6.0, it was added to a HiTrapS anion exchange column and eluted in 0-0.5M NaCl. The GM-CSF bonding activity was detected using ELISA, the active fraction was collected and dialysis was performed using 20 mM Tris HCl pH 9.0. This was added to a HiTrapQ anion exchange column, eluted in the /5 same manner, and dialysis was again performed on the active reaction using 10 mM ammonium acetate. The freeze-dried product was dissolved in a PBS/0.1% NP40 solution and added to a Superose12 gel filtration column. After elution, dialysis was performed on the active fraction using 20 mM TrisHCl pH 9.0 and the product was added to a ResourseQ

column. After dialysis was performed on the active fraction using 20 mM ammonium acetate pH 6.0, the product was added to a ResourseS anion exchange column. After elution, the active fraction was recovered. The protein purity at the various stages of this process was examined using SDS-PAGE.

[0032] (11) GM-CSF Bonded Protein Detection Using Far Western Blotting and Isotope-Labeled GM-CSF

Electrophoresis was performed in a non-reduction state on the BALF or blood serum of the IPAP patients and the product was transferred to a polyvinylidene fluoride film. After blocking the film in a buffer containing albumin, it was reacted with isotope-labeled GM-CSF. After rinsing the film, detection was performed using autoradiography.

[0033] II. Results

(1) Suppression of GM-CSF-Dependent Cell Propagation in IPAP BALF

The monocyte and TF-1 cell cultivation in the presence of 1 ng/ml GM-CSF was inhibited by IPAP BALF. The cultivation inhibition was dependent on the BALF concentration and was not observed when IL-3 was substituted for GM-CSF. The cultivation inhibition in 11 IPAP patients, 2 secondary PAP patients and 10 healthy subjects was examined. The effect was only observed in the IPAP (FIG 1). The monocyte propagation rate when cultivated in 1 ng/ml GM-CSF was 5% or less in the presence of 100 μg/ml IPAP BALF. In contrast, it was 95% or more in the presence of the BALF of healthy subjects. These results indicate that IPAP BALF specifically inhibits GM-CSF.

[0034] (2) Antagonistic Inhibition of GM-CSF and TF-1 Cell Bonding in IPAP BALF

In order to determine whether GM-CSF activity inhibition by IPAP BALF occurs before or after bonding of the GM-CSF to its receptors, the inhibition of radioactive iodide-labeled GM-CSF bonding to TF-1 cells by IPAP BALF was examined. As shown in FIG 2, the inhibition depends on the concentration of IPAP BALF. After suspending TF-1 cells in IPAP BALF, bonding inhibition was not observed even when thoroughly rinsed and reacted with radioactive iodide-labeled GM-CSF. The IPAP BALF acted on the receptors and not only inhibited bonding but also action on the GM-CSF itself.

[0035] (3) Antagonistic Inhibition of GM-CSF-Specific Antibodies in IPAP BALF

Bonding to a specific monoclonal antibody of GM-CSF (23B6) is inhibited by IPAP BALF. As shown in FIG 3, the inhibition was observed in the 11 IPAP patients but not observed in the two secondary PAP patients or the three healthy subjects. IPAP BALF does not inhibit bonding to the specific antibodies of TNFα, IL6 or MCP-1. An increase in the inhibition rate (%) depends on the concentration of IPAP BALF.

[0036] (4) Presence of GM-CSF Bonding Factor in IPAP BALF

From these results, there appears to be a factor bonding to the GM-CSF in IPAP BALF. After reacting radioactive iodide-labeled GM-CSF with IPAP BALF, chemical cross-linking was performed using disuccinimidylsuberate (DSS). When reduction SDS-PAGE is performed,

specific bands were obtained in 39 kD and 41 kD (FIG 4, Lane c), but no bands were obtained in the BALF of the healthy subjects (FIG 4, Lane b). When radioactive iodide-labeled GM-CSF was reacted with IPAP BALF in the presence of an excess amount of non-radioactive GM-CSF, this is weakened significantly (FIG 4, Lane d) which means there is specific bonding to GM-CSF. As a result, the presence of a substance bonding to GM-CSF in IPAP BALF has been confirmed.

[0037] (5) Purification and Properties of GM-CSF-Bonded Proteins in IPAP BALF

Because IPAP BALF is rich in phosphatide, when the BALF was extracted using n-butanol to separate out the lipids from the proteins, the GM-CSF bonding activity was confirmed in the aqueous layer. The fractions were passed through the various stages in the Mono S, Mono Q, Superose 12, Resourse Q and Resourse S columns, and a single Mw 180,000 band was obtained using an SDS-PAGE silver dye.

[0038] [Table 1]

Heat For 10 Minutes at 100°C	Inactive
Heat For 30 Minutes at 55°C	Stable
Chloroform, Methanol Processing	Inactive
n-Butanol Processing	Stable
Tripsin Processing	Stable
Protease K Processing	Stable
Lysozyme Processing	Stable
EDTA Processing	Stable
2-Mercaptoethanol Processing	Stable
pH 4 30 Minutes	Stable
pH 11 30 Minutes	Stable
Molecular Weight (SDS-PAGE)	
Reduced	57 kD
Non-Reduced	180 kD

[0039] The refined proteins obtained in the detection process (10) using far western blotting (11) bonded specifically to GM-CSF.

This was confirmed in a far western blotting test using isotope-labeled GM-CSF. /6

[0040] Working Example 2

The GM-CSF bonded proteins obtained in Working Example 1 were tested in the following manner to confirm the presence of the autoantibody.

(1) NH_2 Terminal Amino Acid Analysis

Of the two proteins obtained from reduction electrophoresis of the refined proteins, 57 kD and 28 kD, when protein 57 kD was analyzed using NH_2 terminal amino acid, there was a 100% match of the NH_2 terminal 20 residues with the human immunoglobulin H strand.

(2) Confirmation of Antibody

When the immunoglobulin component was extracted from IPAP BALF using recombinant protein A, the GM-CSF bonding activity was confirmed in the immunoglobulin fraction. It was clear from this that the GM-CSF bonded protein in the IPAP BALF was the GM-CSF autoantibody.

(3) Isotype Determination

The autoantibodies were confirmed as mainly IgG by the ELISA method.

[0041] Working Example 3 (Confirmation of Presence in Blood Serum)

The anti-GM-CSF autoantibody was confirmed in the blood serum of all IPAP patients (5 patients) using the far western blotting method with isotope-labeled GM-CSF.

[0042] Working Example 4

Anti-GM-CSF Autoantibody Detection Using Far Western Blotting and ^{125}I-GM-CSF

(Method) Blood serum from IPAP patients and healthy subjects was diluted to a total protein amount of 1 mg/mk, one part 10% TCA (trichloroacetic acid) was added over ten minutes to precipitate out the proteins, 2-15% gradient polyacetylamide gel electrophoresis (30 mA constant current, approximately 2 hours) was performed, and transfer to PVDF film was performed (75 minutes at 12 v constant voltage). After solidifying the film for one minute in 10% acetic acid and 50% methanol, the film was allowed to stand overnight at 4°C in a 1% bovine albumin/phosphoric acid buffer solution (pH 7.2). The next day, after adding the film to a vinyl bag containing 0.25 μCi ^{125}I-GM-CSF/phosphoric acid buffer solution (pH 7.2), it was allowed to stand for one hour at room temperature. The film was then removed, rinsed four times in 0.1% Tween 20/phosphoric acid buffer solution, and air dried. Finally, autoradiography was performed on the film.

[0043] (Results) The results are shown in FIG 5 through FIG 7. After performing protein electrophoresis in the upper level column in FIG 5 through FIG 7, the transferred film was dyed with Coomassie Brilliant Blue. In the lower column, the same film was reacted with ^{125}I-GM-CSF to obtain an autoradiography pattern. In screening performed on 11 IPAP patients, 2 secondary PAP patients (FIG 5), 20 healthy patients (FIG 6) and 15 patients with other lung diseases (FIG

7), specific bands with a molecular weight of 180 kD were confirmed only in the IPAP patients.

[0044] Working Example 5

Measurement of the Anti-GM-CSF Autoantibody Using the ELISA Method

(Method) First, 1 μg/ml of recombinant GM-CSF (R&D Co., Ltd.) was added 50 μl at a time to an ELISA plate with 96 wells (NUNC Co., Ltd.). After covering and sealing the plate, it was allowed to stand overnight at 4°C. After rinsing the plate five times in PBS/0.1% Tween 20, 200 μl of 1% BSA/PBS solution was added to the wells and blocking was performed for one hour at room temperature. After absorbing and discarding the liquid, 50 μl diluted patient serum and healthy subject serum as a control were added to the wells and the reaction was continued for another hour at room temperature. The anti-GM-CSF autoantibody was bonded to the GM-CSF. After rinsing the plate five times in PBS/0.1% Tween, 50 μl of 0.3 μg/ml peroxidase-labeled anti-human IgG rabbit antibodies (Dako Co., Ltd.) was added. After conducting a reaction for one hour at room temperature, the plate was again rinsed five times in PBS/0.1% Tween. After adding 50 μl of TMB solution (Scytek Co., Ltd) to each well and conducting a reaction for 15 minutes, 50 μl of STOP solution (Scytek Co., Ltd) was added to stop the reaction. Finally, the absorptivity at 450 nm for each well was measured using an ELISA reader.

[0045] (Results) The results are shown in FIG 8. The OD value was 0.49 to 1.10 for the IPAP serum, 0.12 to 0.16 for the healthy subject serum, 0.15 to 0.19 for the secondary PAP serum, and 0.13 to 0.14 for the congenital PAP serum. In other words, the values for the idiopathic pulmonary alveolar proteinosis serum were significantly higher ($p < 0.01$). GM-CSF has an affinity for serum albumin. For some reason, the reaction with the serum from the healthy subjects was weakened and low-level OD values were indicated. Because bands for 180 kD antibodies could not be detected in these samples using the far western blotting method, the reaction is believed to be non-specific.

[0046] Working Example 6

Measurement of the Anti-GM-CSF Autoantibody Using the Passive Condensation Method

(Method) The beads used to couple the GM-CSF were Polybead Microparticles (Polysciences Co., Ltd.). A glutalaldehyde kit (Cat. #1954) was used in the reaction. The beads were activated in accordance with the kit instruction manual, and 500 ml of % bead solution and 100 mg of recombinant GM-CSF were allowed to stand overnight at room temperature. After centrifugation 15,000 times in six minutes, the supernatant was extracted, suspended in a 0.2 M ethanol amine solution, and reacted for 30 minutes. After centrifugation 15,000 times in six minutes, the supernatant was extracted, the BSA solution from the kit was added, and blocking was performed. In this method, 50% of the GM-CSF was coupled. Finally, 50 ml of 0.05% bead solution and 10 ml of patient and healthy subject

serum diluted 300 times was added to a 96-hole round-bottomed plate, reacted for 24 hours, and observed with the naked eye for hemagglutination.

[0047] (Results) The results are shown in Table 2. Compared to the 38 of 40 healthy human subjects who were negative, the blood serum in 24 of 25 idiopathic pulmonary alveolar proteinosis patients was hemagglutinated. The blood serum of the four secondary pulmonary alveolar proteinosis patients and the two congenital pulmonary alveolar proteinosis patients was free of hemagglutination. /7

[0048] [Table 2]

	Latex Agglutination Reaction	
	Positive	Negative
Idiopathic Pulmonary Alveolar Proteinosis	24	1
Non-Idiopathic Pulmonary Alveolar Proteinosis	2	44
Secondary Pulmonary Alveolar Proteinosis	0	4
Congenital Pulmonary Alveolar Proteinosis	0	2
Healthy Persons	2	38

[0049] Working Example 7

Measurement of the Anti-GM-CSF Autoantibodies Using a Bioassay with TF-1 Cells:

(Method) In this method, the autoantibody itself was not observed. Instead, TF-1 cell propagation in the weakened activity of the GM-CSF neutralized by the autoantibody was measured as an indicator. The TF-1 cells were provided by Dr. Toshihiro Kitamura at the Institute of Medical Science [University of Tokyo]. The cells were a tumor cell strain whose propagation depended on GM-CSF. The cultivation occurred in a 96-hole flat-bottomed plate sterilized using gamma radiation. Ten thousand TF-1 cells were suspended in a 100 ml RPMI 1640 culture

containing 1 ng/ml recombinant GM-CSF and 0.25% blood serum from the healthy subjects or the patients (and including 10% vituline serum). This was cultivated for four days at 37.5°C in the presence of carbon dioxide gas. Then, 10 ml of 5 mg/ml MTT solution was added to each one of the wells and cultivated for another three hours. Because the MTT was soluble and entered the cells, 100 ml of 0.04N hydrochloric acid isopropanol solution was added, the pigment was thoroughly dissolved, and the absorptivity at 560 nm measured using an ELISA reader.

[0050] (Results) The TF-1 cells propagated extremely well in the presence of 1 ng/ml GM-CSF. The number of cells after four days of cultivation had quadrupled. Because the IPAP blood serum contained the anti-GM-CSF autoantibody, the added GM-CSF effect was neutralized and cell propagation was not observed. When the MTT reagent had entered the cells, it was metabolized and changed into a hydrophobic pigment. When the absorptivity of the pigment was measured, as shown in FIG 9, the OD value was 0.37 to 0.69 for the IPAP serum, 1.3 to 1.4 for the healthy subject serum, 1.0 to 1.2 for the secondary PAP serum, and 0.9 to 1.2 for the congenital PAP serum. In other words, the values for the idiopathic pulmonary alveolar proteinosis serum were significantly lower ($p < 0.01$).

[0051] [Effect of the Invention]

The autoantibody of the present invention is an autoantibody for GM-CSF known to contribute to various inflammatory diseases in the human body. By measuring this autoantibody, these inflammatory diseases can be diagnosed. It can especially make blood serum

diagnoses of idiopathic pulmonary alveolar proteinosis more sensitive and specific.

[Brief Explanation of the Drawings]

[FIG 1] A diagram showing the action of TF-1 cells (2×10^5) propagating in BALF (100 μm/ml).

[FIG 2] A diagram of the IPAP antagonistic action on the bonding of GM-CSF and TF-1 in BALF.

[FIG 3] A diagram showing the inhibition of anti-GM-CSF monoclonal antibodies on GM-CSF in BALF.

[FIG 4] A diagram showing SDS-PAGE results for proteins bonded to GM-CSF in IPAP BALF via a cross-linking agent. Lane (a) shows the results for radiolabeled GM-CSF alone, Lane (b) the results from the BALF of healthy persons, Lane (c) the results from the BALF of IPAP patients, and Lane (d) the results from radiolabeled GM-CSF in IPAP BALF in the presence of a surplus amount of non-radiolabeled GM-CSF.

[FIG 5] A diagram showing the results from the far western method using the blood serum from IPAP patients (1-11), secondary PAP patients (12, 13) and ^{125}I-GM-CSF.

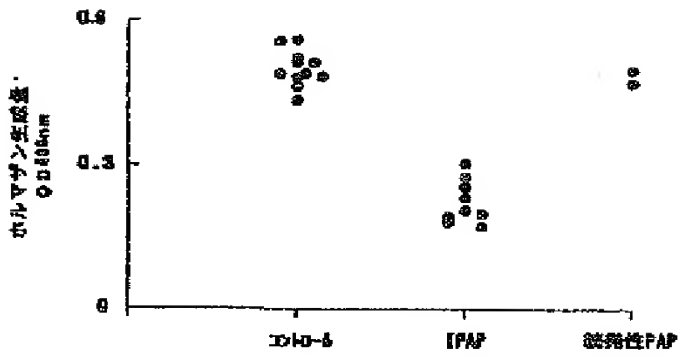
[FIG 6] A diagram showing the results from the far western method using the blood serum from healthy persons (14-33) and ^{125}I-GM-CSF.

[FIG 7] A diagram showing the results from the far western method using the blood serum from patients with lung diseases other than PAP (34-36) and ^{125}I-GM-CSF.

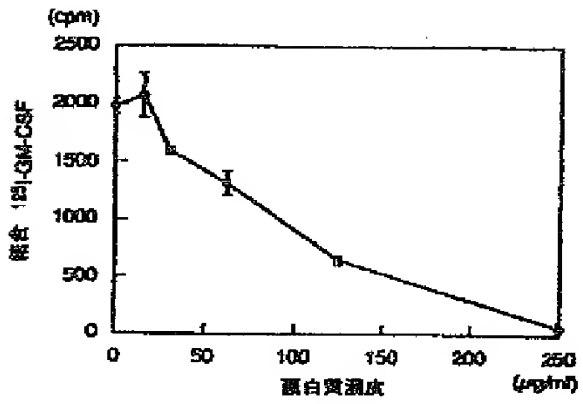
[FIG 8] A diagram showing the results of anti-GM-CSF autoantibody measurements in the blood serum of patents and healthy persons using ELISA.

[FIG 9] A diagram showing the results of anti-GM-CSF autoantibody measurements in the blood serum of patents and healthy persons using a bioassay with TF-1 cells.

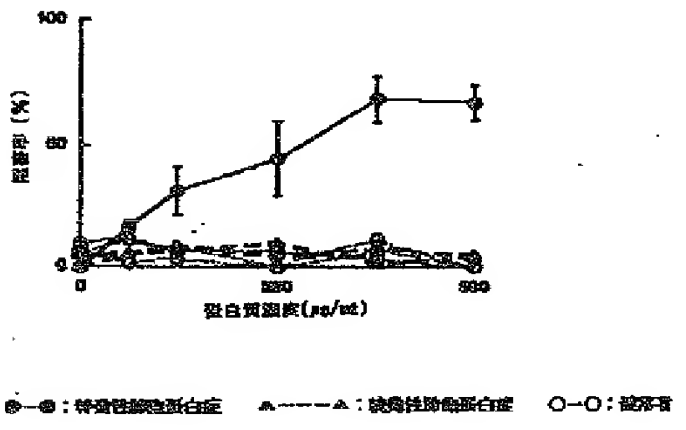
[Figure 1]



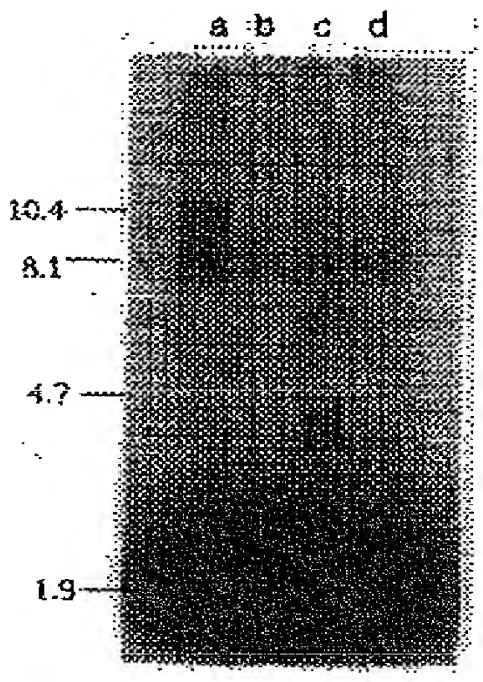
[Figure 2]



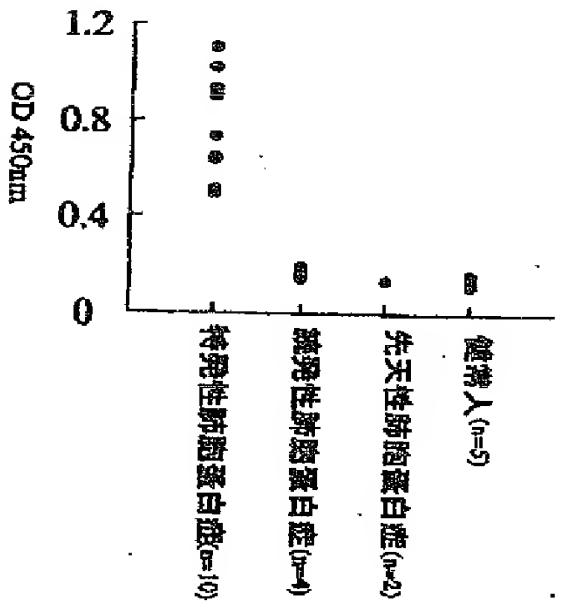
[Figure 3]



[Figure 4]



[Figure 8]



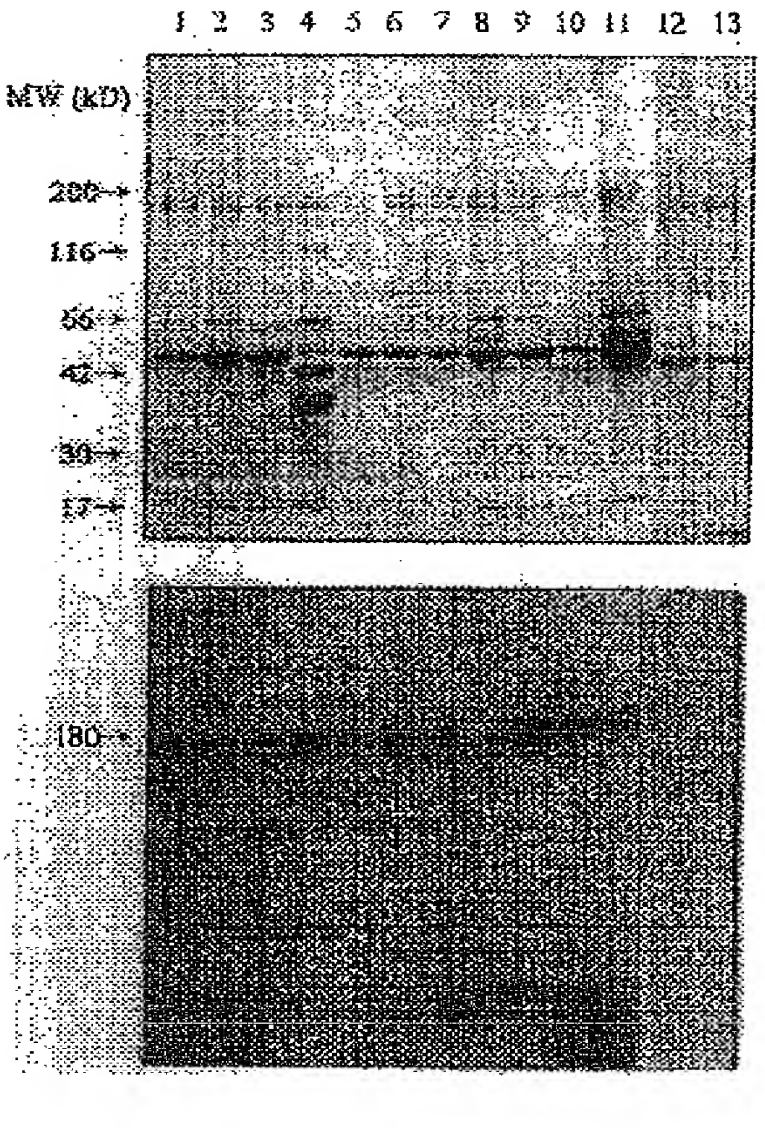
[FIG 1]
{x-axis} Control IPAP Secondary PAP
{y-axis} Amount of Hormones Generated

[FIG 2]
{x-axis} Protein Concentration
{y-axis} Bonding

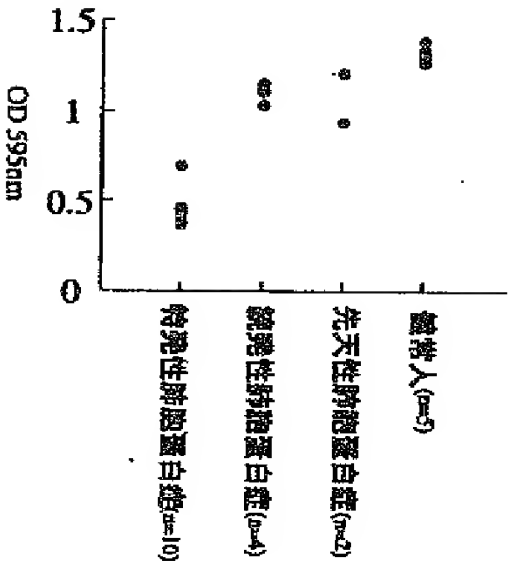
[FIG 3]
{x-axis} Protein Concentration
{y-axis} Inhibition Rate (%)

[FIG 8]
Healthy Persons (n=5)
Secondary Pulmonary Alveolar Proteinosis (n=2)
Congenital Pulmonary Alveolar Proteinosis (n=4)
Idiopathic Pulmonary Alveolar Proteinosis (n=10)

[Figure 5]

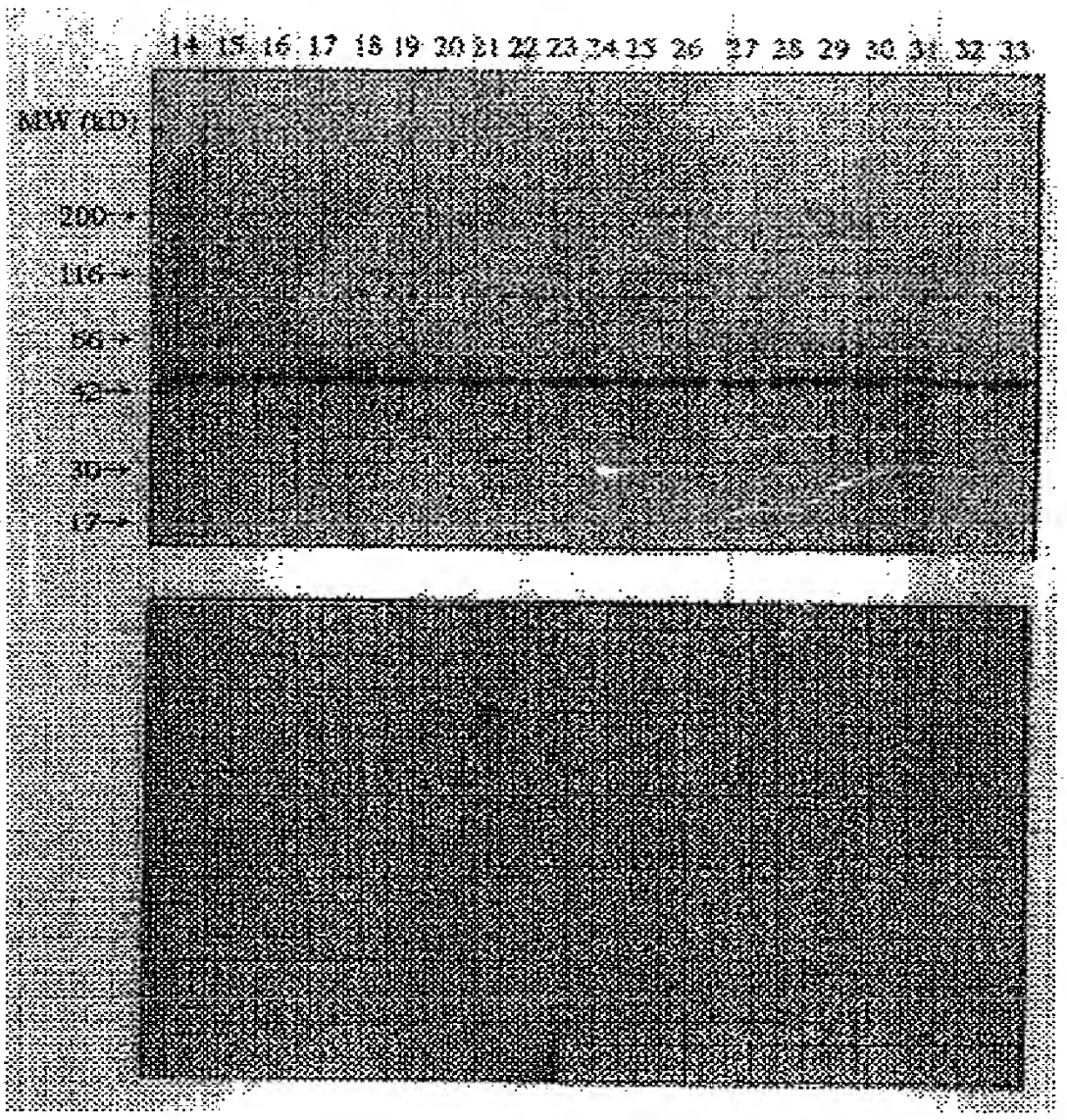


[Figure 9]



[FIG 9]
Healthy Persons (n=5)
Secondary Pulmonary Alveolar Proteinosis (n=2)
Congenital Pulmonary Alveolar Proteinosis (n=4)
Idiopathic Pulmonary Alveolar Proteinosis (n=10)

[Figure 6]


1+ 15 16 17 18 19 30 21 22 23 24 25 26 27 28 29 30 31 32 33
MW (kD):
200→
116→
66→
42→
30→
17→

[Figure 7]